104 (2025) 105015

Contents lists available at ScienceDirect

Poultry Science

journal homepage: www.elsevier.com/locate/psj

Full-Length Article

Chinese herbal medicine improves antioxidant capacity of chicken liver at high stocking density involved gut-liver microbiota axis based on multi-omics technologies☆

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ARTICLE INFO

Keywords:
High-density farming
broiler
multi-omics
cholesterol
liver microbiome

ABSTRACT

Traditional Chinese Medicine (TCM), such as artemisinin, berberine and proanthocyanidin, has been considered an effective additive for broiler production. High density farming (HDF), which is the primary modern mode of chicken production, is associated with animal health problems. This work aimed to evaluate the effects of dietary TCMs (dihydroartemisinin, hydrochloride, and oligomeric proanthocyanidins) on improving the antioxidant capacity of chickens under HDF and their underlying mechanisms. A total of 360 Wuding chickens (134-day-old) were divided into five experimental groups: one normal stocking density (8 birds/m^2, control group) and four high stocking density (16 birds/m^2), with six replicates for each group. For four HDF groups, one group was fed the basal diet, and the other three groups were fed the basal diet supplemented with 80 mg/kg dihydroartemisinin, 600 mg/kg berberine hydrochloride, and 250 mg/kg grape oligomeric proanthocyanidins, respectively. HDF increased malondialadehyde level, but decreased superoxide dismutase, glutathione and glutathione peroxidase levels in the liver of broiler; however, dietary TCMs apparently alleviated this attenuation. Dietary TCMs significantly decreased the expression of genes involved in cholesterol synthesis in the liver and the levels of tripepides in the intestine of the HDF chickens. Meanwhile, dietary TCMs significantly altered the composition of the liver microbiome in the HDF chickens, expressing by reduced *Pseudomonas* but enriched *Bradyrhizobium*. The gut microbiota of the HDF chickens was also altered following dietary TCM administration, with a decreased abundance of *Microbacter margulisiae* and an increased abundance of acetate synthesis genes. Association analysis of the multi-omics results revealed negative correlations between liver cholesterol synthesis and antioxidant factors that could be regulated by gut microbiota-produced short-chain fatty acids. Furthermore, alleviating of oxidative stress by dietary TCMs also showed significant correlations with the liver microbiome, which could be mediated by tripeptides produced by the gut microbiota. These results indicated that dietary TCM is beneficial in improving antioxidant defenses in HDF chickens and interpreted the mechanisms of action of TCM from the perspective of modern science.

☆ **Scientific section: Metabolism and Nutrition**

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https://doi.org/10.1016/j.psj.2025.105015
Received 20 December 2024; Accepted 10 March 2025

Introduction

To meet the growing global demand for affordable chicken meat, the application of high-density farming (HDF) has become a prominent method as it can maximize space utilization and production (Gržinić et al., 2023). However, HDF also raises concerns about animal welfare and environmental sustainability due to the close proximity of chickens in high-density farms (Mace et al., 2022). A key issue that arises in these HDF environments is the increased risk of oxidative stress in the chickens (Nasr et al., 2021). The crowded conditions of HDF systems provide a variety of stressors that can contribute to increased oxidative stress in chickens (Sugiharto, 2022). The consequences of oxidative stress in high-density chicken production can be significant, including reduced growth, impaired immune function and increased susceptibility to disease (Son et al., 2022). In addition, high levels of oxidative stress can lead to poor meat quality, reduced egg production, and even increased mortality rates within the flock (Akinyemi and Adewole, 2021). By addressing strategies to mitigate oxidative stress, the welfare and productivity of chickens in HDF systems can be improved, leading to more sustainable and responsible poultry production.

Traditional Chinese medicine (TCM) is important for human and animal health (Still, 2003). Incorporating TCM into chicken husbandry strengthens the immune system and promotes overall health and resilience (Jamil et al., 2022). For example, artemisin is a sesquiterpene lactone endoperoxide isolated from the traditional Chinese herbal medicine *Artemisia annua*. Its antioxidant and antimicrobial effects help mitigate stress responses during disease challenges, which is crucial for maintaining production levels in affected flocks (Sharma et al., 2024). In addition, berberine (a quaternary ammonium alkaloid) isolated from the Huanglian is the main effective ingredient in Huanglian's antibacterial activity. Research has demonstrated that berberine alters the compositions and functions of the gut microbiota, which can improve overall gut health and performance in broilers (Zhu et al., 2021). Moreover, proanthocyanidins are a general term for a large class of polyphenolic compounds widely present in plants, which have been indicated to significantly reduce oxidative stress in broilers under pathogenic infections (Wang et al., 2008). Therefore, TCM remedies used in chicken farming often contain a variety of antioxidant-rich ingredients that play a supportive role in restoring harmony and optimal function.

TCM has also been shown to have several beneficial effects on the gut microbiota of poultry, particularly in enhancing intestinal health and overall performance. TCM supplements in poultry farming increased the abundance of beneficial genera, such as *Lactobacillus*, while decreasing potentially harmful bacteria like Bacteroides and Firmicutes (Liu et al., 2023a). By promoting a more favorable gut microbiota, TCM can enhance nutrient absorption of poultry, leading to better growth outcomes (Zhou et al., 2023). Moreover, the regulation of the gut microbiota by TCM can indirectly support liver health by reducing inflammation and preventing the translocation of harmful substances into circulation (Fu et al., 2021). Interpreting the principles of TCM in poultry management practices from the perspective of modern science is considered crucial to deeply embedding TCM in poultry production systems.

Dietary supplementation with TCMs has been linked to enhance antioxidant capacity in poultry (Liu et al., 2023b). By improving the antioxidant defense mechanisms within the body, TCM can help mitigate stressors that poultry face, such as environmental challenges (Liu et al., 2024). However, previous studies have not clearly explained the detail mechanisms of TCMs in enhancing antioxidant defenses in broilers, particularly under HDF stress. The gut-liver axis refers to a bidirectional relationship between the gut microbiota and the liver, which is an important pathway for gut microbiota to affect the host (Albillos et al., 2020). Research has indicated that TCM supplements can prevent harmful substances from entering the bloodstream and affecting liver function by regulating the gut microbiota (Luo et al., 2024). Based on these findings, we provided a hypothesis that dietary supplementation could alleviate oxidative stress in broilers' livers under HDF via the gut-liver microbiome axis. The objectives of this work were i) to determine whether HDF can induce weaker antioxidant capacity in chickens and ii) to evaluate the effects of dietary TCMs on improving the antioxidant capacity of chickens under HDF and their underlying mechanisms. Three TCM extracts, artemisin isolated from *Artemisia annua* L., berberine isolated from Huanglian, and proanthocyanidin isolated from grape seeds, were selected as dietary supplements for the HDF chickens. By combining multi-omics datasets, including liver transcriptome, liver microbiome, intestinal metabolome, and gut microbiota, the mechanisms of dietary TCMs to improve the antioxidant capacity of chickens under HDF through different pathways of the gut-liver axis were revealed. The results of this study contribute to exploring the approach to overcome the weak antioxidant capacity in high-density broiler farming and highlight TCMs as an effective additive.

Material and methods

Experimental chicken, diet, and sample collection

The chicken experiment in this study was approved by the Animal Care and Use Committee of Yunan Agricultural University (No. 202210012). A total of 360 Wuding chickens (134 days old and similar body weight), a representative local chicken breed in Yunnan Province, Southwest China, were divided into five experimental groups: one normal stocking density (8 birds/m^2, control group, CG) and four high stocking density (16 birds/m^2), with six replicates (henhouse) for each group. For four HDF groups, one group was fed the basal diet (HG), and the other three groups were fed the basal diet supplemented with 80 mg/kg dihydroartemisinin (HA group), 600 mg/kg berberine hydrochloride (HB), 250 mg/kg grape oligomeric proanthocyanidins (HP), respectively. All broilers were reared in a standardized environment with a temperature maintained at 23°C and humidity ranging from 45 % to 55 %. The basal diet (Table S1) was formulated based on NY/T 3645-2020, and the duration of the experiment was 10 weeks.

At the end of the experiment, 12 broilers/group (2 broilers/replicate) were selected and sacrificed to collect their blood, liver, and cecal digesta samples for further analysis. The blood was centrifuged. Subsequently, the supernatant was carefully collected to obtain the serum, which was then stored at −80°C. The liver (which was cut into small segments) and cecal digesta samples was also stored at −80°C for further analysis

Measurement of anti-oxidative indices, cholesterol levels, and SCFAs contents

The enzyme activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and the levels of malondialadehyde (MDA), glutathione (GSH), and cholesterol in the liver and serum samples were measured with commercial kits (Jiancheng Institute of Bioengineering, Nanjing, China) using a spectrophotometer (Jingke Company, Shanghai, China). According to a previous study, the contents of SCFAs in the cecal digesta samples were measured by targeted metabolomics technology (Hu et al., 2024b).

RNA extraction and RNA-seq of the liver

Total RNA was extracted from HDF chicken livers using the Trizol reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After quality control by agarose gel electrophoresis, the mRNA was enriched on oligo(dT) beads. Sequencing libraries (paired-end 150 bp) were constructed using the TruSeqTM RNA sample preparation kit (Illumina, San Diego, CA) and sequenced on the Illumina NovaSeq 6000 platform at Shanghai BIOZERON Co., Ltd (Shanghai, China). Quality control of the raw reads was performed using fastp software with

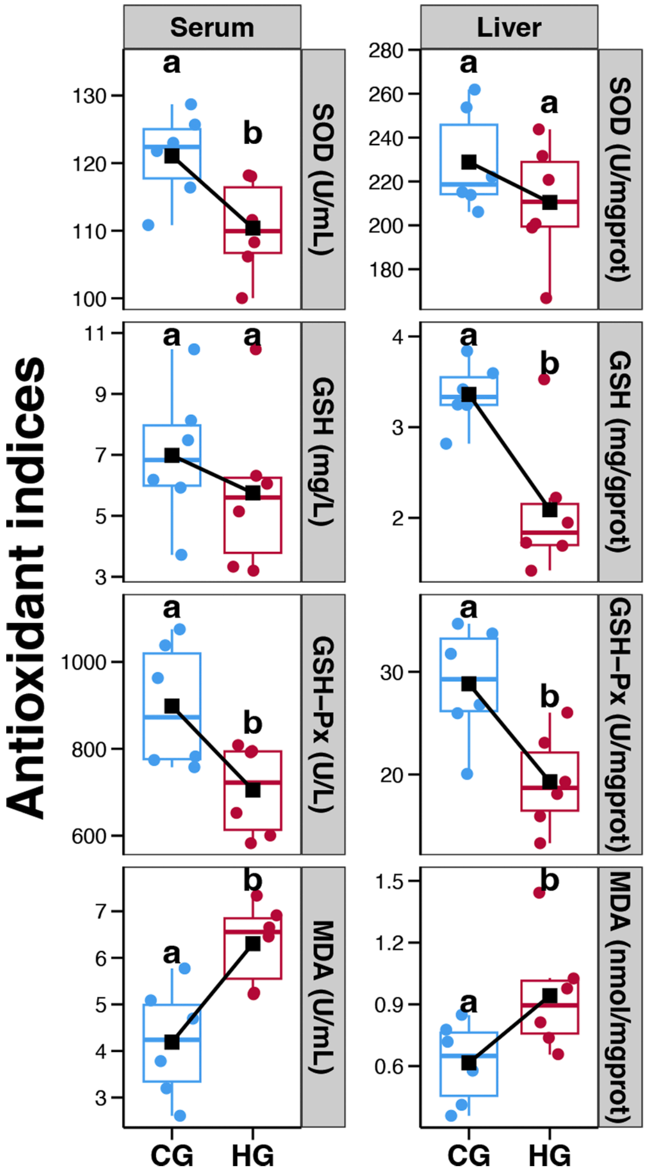

Fig. 1. Differences in serum and liver antioxidant indices between Wuding chickens on normal and HDF. Different lower case letters above the boxes of each sub-figure represent significant differences between different groups based on the Wilcox rank sum test ($p < 0.05$).

parameters of unknown nucleotides less than 10 % and Q20 > 50 % (Chen et al., 2018). Bowtie2 (Langmead and Salzberg, 2012) was used to map reads to the ribosomal RNA (rRNA) database, and the rRNA-mapped reads were removed. The processes of reference genome mapping and gene expression calculation were performed by HISTA2 (Kim et al., 2015) and RESM (Li and Dewey, 2011), respectively. Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al., 2014) with a fold change threshold greater than 2 and a fasle discovery rate (FDR)-adjusted p-value of less than 0.5. KEGG annotation of the DEGs was then obtained from the reference genome, and enrichment analysis was performed using clusterProfiler 4.0 (Wu et al., 2021).

Sequencing of the liver microbiome

Total DNA was extracted from the Wuding chicken liver tissue using an E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, Norcross, GA). The V3-V4 regions of the bacterial 16S rRNA gene were amplified from each extracted DNA using primers 341F and 806R with adapter sequences and barcodes attached to the end of the reverse primer (Berg et al., 2012). The sequencing libraries were sequenced on an Illumina Novaseq6000 platform with a 250 bp paired-end strategy at BIOZERON Biotech. Ltd in Shanghai, China. After sequencing, the raw reads were assigned to the samples based on their unique barcodes. Then, reads with average Phred scores lower than 20, containing ambiguous bases or having homopolymer runs higher than 8, having mismatches in the primers, and having sequence lengths shorter than 250 bp were removed (Nicholas et al., 2013). Paired-end reads with an overlap greater than 10 bp were assembled according to their overlap sequence. Operational taxonomic units (OTUs) were clustered with a 97 % similarity cutoff using UPARSE version 10 (Edgar, 2013), and chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). Singletons (the number of a particular OTU was one) were discarded, and all remaining OTUs were assigned to a taxonomy using the SILVA database (release 138) (Yilmaz et al., 2014).

Non-targeted metabolomics of the cecal digesta

For metabolite extraction, 100 mg of cecal digesta from each HDF chicken was snap-frozen in liquid nitrogen immediately after dissection. The samples were then homogenized with 200 μL water and five ceramic beads. The homogenized solution was resuspended with 800 μL of pre-chilled methanol/acetonitrile (1:1, *v:v*). The mixture was centrifuged for 15 min (14,000 g and 4°C), and the supernatant dried in a vacuum centrifuge. The supernatant was re-dissolved in 100 μL acetonitrile/water (1:1, *v:v*) and injected onto a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) for metabolite separation and detection. The detailed process of UHPLC-MS/MS was consistent with a previous study (Rathod et al., 2019). The peak of metabolites was selected by the XCMS software based on the raw MS data with the following parameters: centWave m/z = 10 ppm, peak width = c (10, 60), and prefilter = c (10, 100). For peak grouping bw = 5, mzwid = 0.025, and minfrac = 0.5. Compound identification of metabolites was performed by comparison of accuracy m/z value (<10 ppm) and MS/MS spectra with an in-house database established with available authentic standards. Comparisons of metabolites between the HDF chickens with and without dietary TCMs were performed. Wilcox rank sum test (p-value < 0.05), VIP value (greater than 1) and a fold change greater than 2 or less than 0.5 were considered to identify differentially abundant metabolites (DAMs).

Sequencing of the gut microbiota

Microbial DNA was extracted from the cecal digesta using the QIAGEN DNA Stool MiniKit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The full length (V1-V9 region) of the bacterial 16S rRNA gene was amplified using primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′), where an eight-base sample-specific barcode sequence was combined in the reverse primer. The detailed amplification and library construction processes were according to a previous study (Gao et al., 2022); thereafter, sequencing was performed on a PacBio Sequel IIe platform at Shanghai Biozeron Biotechnology Co. Ltd (Shanghai, China). PacBio raw reads were processed using SMRT Link Analysis software version 11.0 to obtain demultiplexed circular consensus sequence CCS reads with the following settings: minimum number of passes = 3 and minimum predicted accuracy = 0.99. Reads of inappropriate length (>1000 or <1800 bp) were then removed, and the barcode and primer sequences were clipped using the lima pipeline (Pacific Biosciences demultiplexing barcoded software, https://lima.how/). OTUs were clustered using UPARSE version 10 (Edgar, 2013) with a similarity cutoff of 98.65 % (Johnson et al., 2019). The representative sequences of each OTU were assigned to a taxonomy using the SILVA database (release 138) (Yilmaz et al., 2014). The metagenomics sequencing of cecal digesta from HDF

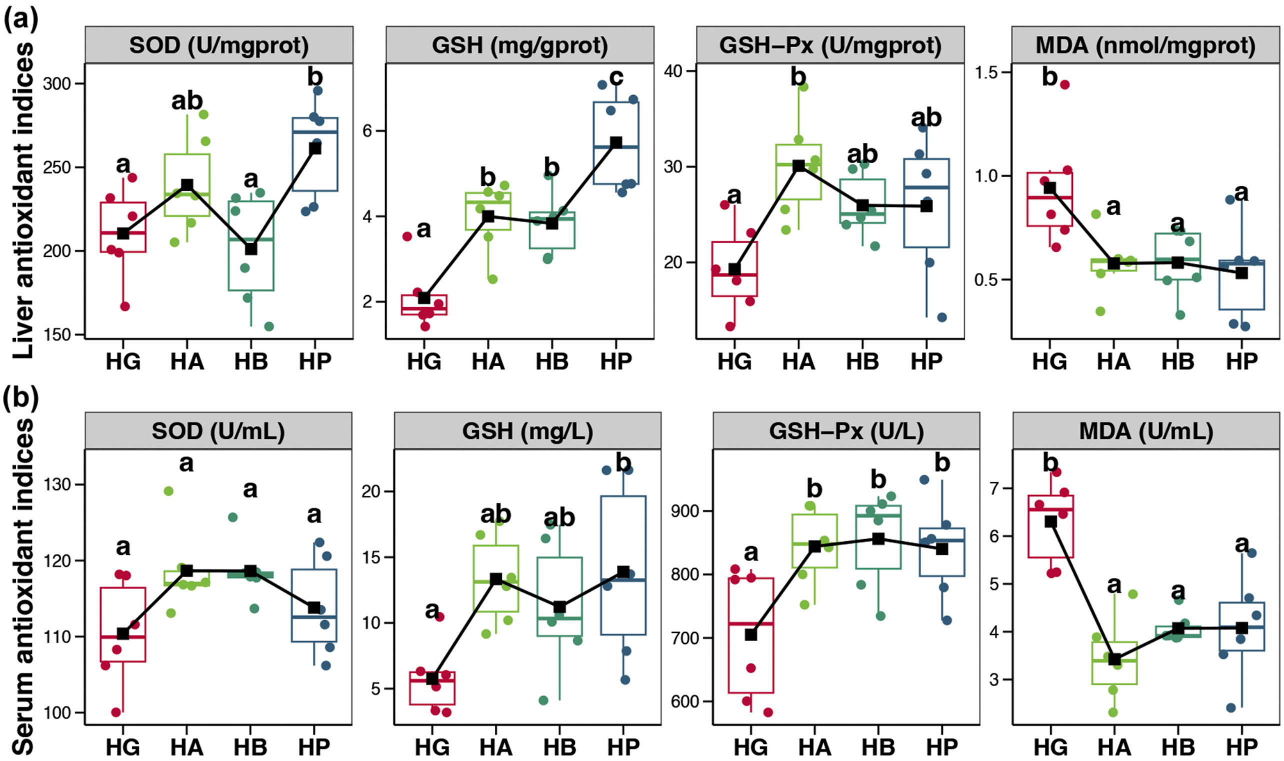

Fig. 2. Differences in antioxidant indices in liver (a) and serum (b) between HDF Wuding chicken with and without dietary TCM. Different lowercase letters above the boxes of each sub-figure represent significant differences between different groups based on Tukey's HSD test ($p < 0.05$).

Wuding chickens was performed in our previous study (Hu et al., 2024a), and the raw sequencing data can be downloaded from the China National GeneBank Sequence Archive (CNSA) of the China National GeneBank DataBase (CNGBdb) with accession number CNP0005172. The annotation of genes related to short-chain fatty acid (SCFA) biosynthesis and amino acid metabolism was obtained based on the KEGG database according to a previous study (Wang et al., 2024).

Statistical analysis

All statistical analyses were performed in R v4.2.2 (Crawley, 2014) and visualized by the 'ggplot2′ (Valero-Mora, 2010) and 'pheatmap' (Kolde and Kolde, 2015) packages. Differences in antioxidant indices, cholesterol levels, and SCFAs contents between the CG and HG samples were estimated by the Wilcox rank sum test, and variations between the different HDF treatments were compared by Tukey's HSD test ("multcomp" package). Principal component analysis (PCA) ("FactoMineR" package) and partial least squares discriminant analysis (PLS-DA) ("mixOmics" package) were performed to evaluate the variations in liver gene expression profiles and intestinal metabolic patterns among the HDF chickens with and without dietary TCMs, respectively. In addition, a Venn diagram was drawn using the VennDiagram package to identify the common DEGs and DAMs in all three TCM-supplemented treatments compared to the HG individuals.

Four alpha diversity indices of the liver microbiome and gut microbiota related to different facets, including richness (Chao1), evolution (Faith's phylogenetic diversity, Faith_pd), diversity (Shannon) and evenness (Pielou's evenness index, Pielou_J) were calculated using the "vegan" package. Variations in alpha diversity indices between the different HDF treatments were also analyzed using Tukey's HSD test. Principal coordinate analysis (PCoA) based on Bray-Curtis distance was performed ('ape' and 'vegan' packages) to assess the effects of dietary TCMs on the composition of the liver microbiome and gut microbiota in the HDF chickens. The Wilcox rank sum test was used to identify the liver bacterial genera and gut bacterial species that had significant differences in relative abundance between the HDF chickens with and without dietary TCMs. The neutral community model was constructed to investigate the assembly mechanism and migration rate of the liver microbiome of the Wuding chickens (Sloan et al., 2016). In addition, variations in the abundance of genes related to SCFA synthesis and amino acid metabolism in the gut microbiota of the HDF chickens with and without dietary TCMs were tested using Tukey's HSD test.

Finally, correlations between liver or serum antioxidant indices, genes related to cholesterol synthesis in the liver, common DAMs in the gut, key bacteria in the liver microbiome, and functional genes involved in SCFAs synthesis in the gut microbiota were analyzed by Spearman's correlation method ("psych" package) to explore potential interactions in the gut-liver axis.

Results

Antioxidant indices of wuding chickens under HDF

Compared to the CG group, the SOD and GSH-Px were significantly decreased in the serum of the HG group (Wilcox rank sum test, $p < 0.05$, Fig. 1); the SOD, GSH and GSH-Px were also significantly decreased in the liver of the HG group (Wilcox rank sum test, $p < 0.05$, Fig. 1). In addition, a significant increase in MDA levels was observed in both the serum and liver of HG individuals compared to the CG samples (Wilcox rank sum test, $p < 0.05$, Fig. 1).

Antioxidant indices of wuding chickens with dietary TCMs

For the liver antioxidant system, the activities of SOD and GSH-Px in the HDF Wuding chickens were significantly increased in the HP and HA groups, respectively (Tukey's HSD test, $p < 0.05$, Fig. 2a). Meanwhile, a significantly higher level of GSH was observed in all three TCM-supplemented groups compared to the HG individuals (Tukey's HSD test, $p < 0.05$, Fig. 2a). For the serum antioxidant system, a significantly higher activity of GSH-Px was found in all three TCM-supplemented

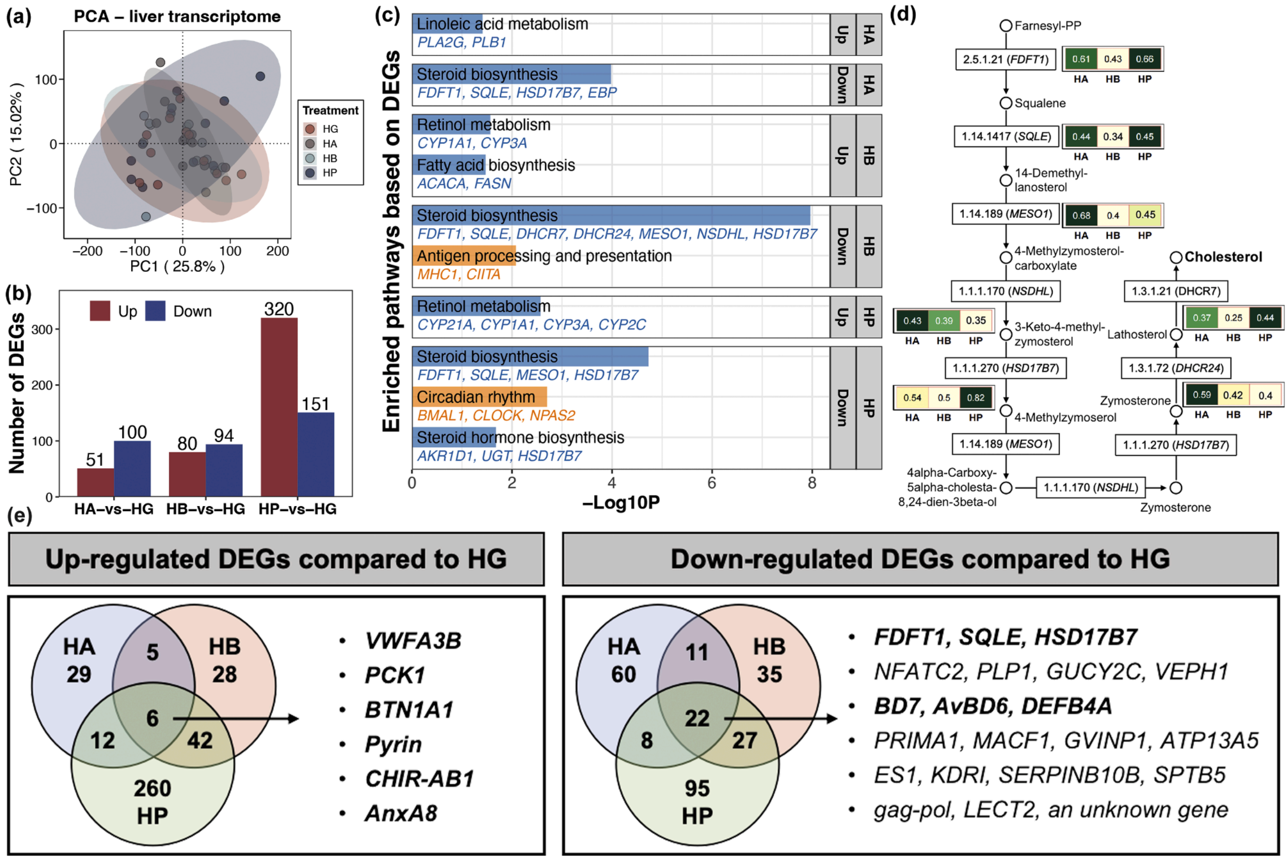

Fig. 3. (a) Principal component analysis (PCA) reveals the variations of gene expression profiles in the liver of HDF Wuding chicken with and without dietary TCM. (b) Number of differentially expressed genes (DEGs) between liver of HDF Wuding chicken with and without dietary TCM. (c) KEGG enrichment analysis of DEGs. Blue and orange bars represent metabolic and signal transduction pathways, respectively. (d) Cholesterol biosynthesis pathway. Heatmap showing the fold change of gene expression level between the liver of HDF Wuding chicken with and without dietary TCMs. (e) Venn diagram showing the common up- and down-regulated DEGs between different comparisons.

groups compared to the HG individuals (Tukey's HSD test, $p < 0.05$, Fig. 2b). The level of GSH was significantly increased only in the serum of the HP individuals compared to the HG samples (Tukey's HSD test, $p < 0.05$), and no significant variation in SOD activity was detected (Fig. 2b). In addition, MDA levels were significantly reduced in both the serum and liver of the Wuding chickens in all three TCM-supplemented groups compared to the HG counterparts (Tukey's HSD test, $p < 0.05$, Fig. 2b). Taken together, dietary TCMs could improve the reduced antioxidant capacity of Wuding chickens under HDF.

Liver transcriptome of wuding chickens

According to the transcriptome results, PCA showed a slight variation in the gene expression profiles of the liver from the HDF Wuding chickens with and without dietary TCMs (Fig. 3a). In addition, several DEGs were identified, with 51, 80 and 320 genes up-regulated and 100, 94 and 151 genes down-regulated in the HA, HB and HP groups, respectively, compared to the HG individuals (Fig. 3b). KEGG enrichment analysis revealed that linoleic acid metabolism pathways were significantly enriched in the HA individuals compared to the HG samples based on up-regulated DEGs ($p < 0.05$, Fig. 3c). The retinol pathway was up-regulated in the HB and HP groups compared to HG samples, fatty acid biosynthesis was also up-regulated in HB individuals (Fig. 3c). Antigen presentation pathways were significantly downregulated only in the HB group, and circadian rhythm and steroid hormone biosynthesis were significantly downregulated only in the HP group compared to the HG individuals ($p < 0.05$, Fig. 3c). Furthermore, the steroid biosynthesis pathway was significantly down-enriched in all three TCM-added groups compared to the HG samples ($p < 0.05$, Fig. 3c). DEGs involved in the steroid biosynthetic pathway were also detected, and all of them were involved in the biosynthesis of cholesterol (Fig. 4d), indicating the inhibition of cholesterol synthesis in the liver of Wuding chickens with dietary TCMs. In addition, 6 and 22 common DGEs were identified in all three comparisons between the HDF Wuding chicken with and without TCM supplementation (Fig. 3e). The six common upregulated DEGs were *VWFA3B, PCK1, BTN1A1, Pyrin, CHIR-AB1*, and *AnxA8*. The common down-regulated DEGs also included some genes involved in cholesterol synthesis (*FDFT1, SQLE*, and *HSD17B7*) and some beta-defensins (*DB7, AvDB6*, and *DEFB4A*).

Liver microbiome of wuding chickens

Based on Illumina sequencing, a total of 720 bacterial OTUs were identified from the Wuding chicken liver, belonging to 8 phyla, 11 classes, 29 orders, 51 families, and 93 genera. The rarefaction and species accumulation curves showed a plateau trend (Fig. S1), suggesting that the data set presented here may reflect an intact Wuding chicken liver microbiome. As the liver microbiome was obtained by Illumina sequencing, the annotation of OTUs at the species level was only 19.03 %, but nearly all the OTUs (98.75 %) were assigned to a genus (Fig. S2a). *Vibrionimonas* (20.48 %) was the most dominant bacterial genus in the Wuding chicken liver microbiome, followed by *Bradyrhizobium* (12.84 %), *Pseudomonas* (12.33 %), *Variovorax* (7.52 %), and *Burkholderia* (6.28 %) (Fig. 4a). For the four calculated alpha diversity indices, only the Pd_faith index showed a significant increase in the TCM-added groups compared to the HG individuals (Tukey's HSD test, $p < 0.05$, Fig. 4b and

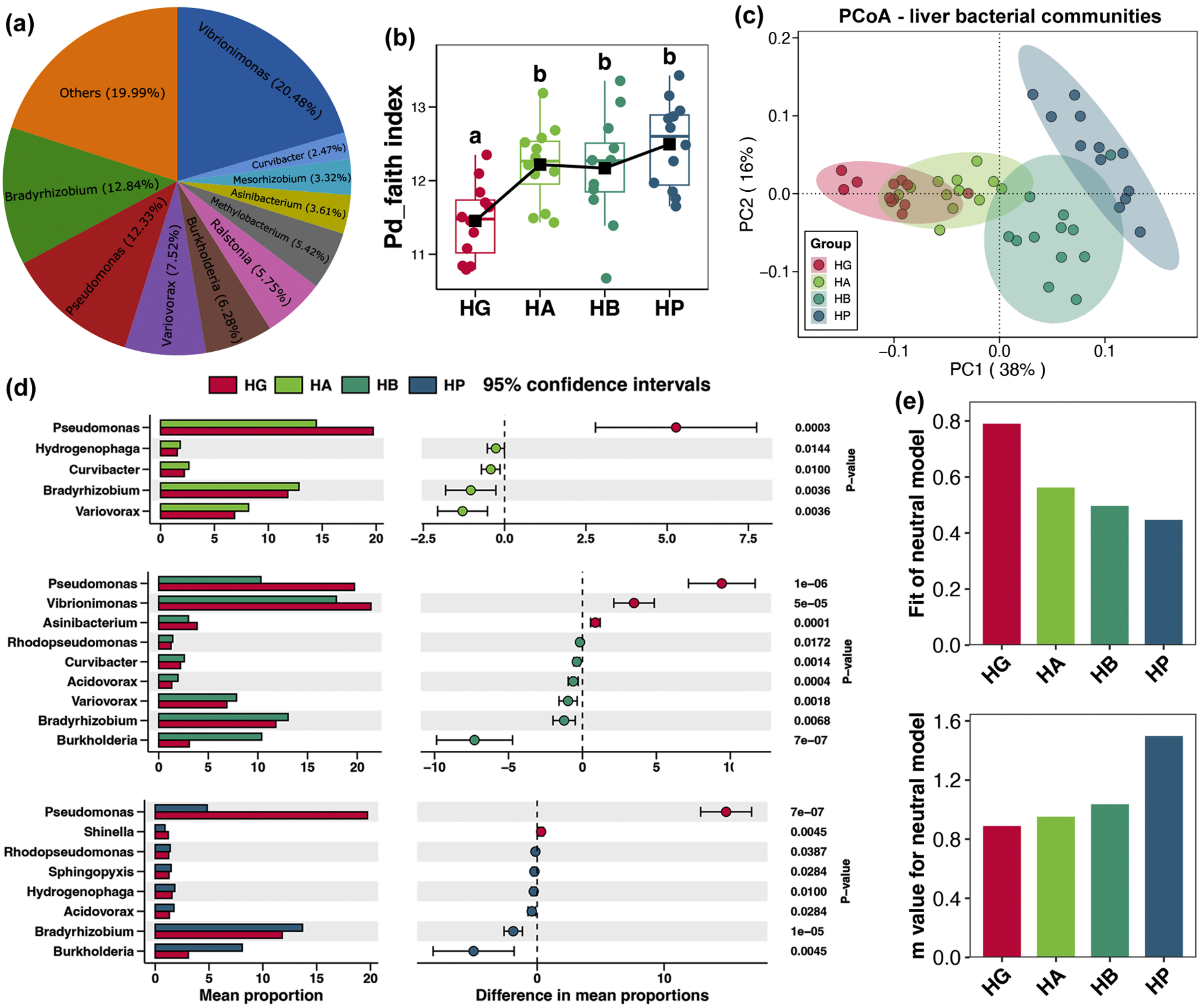

Fig. 4. (a) Pie chart showing the relative abundance of major bacterial genera in the liver microbiome of Wuding chickens. (b) Variations in the Pd_faith index of the liver microbiome of Wuding chickens between different treatments. Different lowercase letters above the boxes represent significant differences between different groups based on Tukey's HSD test ($p < 0.05$). (c) Principal Coordinate Analysis (PCoA) shows the variations in the liver microbiome of HDF Wuding chicken with and without the dietary TCMs. (d) Genera with significant difference in relative abundance (WIlcox rank sum test, $p < 0.05$) in the liver of HDF Wuding chicken with and without dietary TCMs. (e) Bar plot comparing the fit and m-value of neutral models for the liver microbiome of HDF Wuding chicken with and without dietary TCMs.

S2b). In contrast, PCoA revealed clear differences in the composition of the liver microbiome among the different groups, which were individually clustered and separated by the PC1 axis (Fig. 4c). For the specific bacterial genera, *Pseudomonas* and *Bradyrhizobium* were significantly reduced and enriched, respectively, in all three TCM-added groups compared to the HG samples (Wilcox rank sum test, $p < 0.05$, Fig. 4d). In addition, the relative abundance of *Burkholderia* was significantly increased in the HB and HP individuals, and *Variovorax* was more abundant in the HA and HB individuals compared to the HG counterparts (Wilcox rank sum test, $p < 0.05$, Fig. 4d). Furthermore, the neutral model revealed a less stochastic assembly with high migratory ability in the liver microbiome of the HDF Wuding chicken with dietary TCMs (Fig. 4e).

Intestinal metabolites of wuding chickens

Based on the non-targeted metabolome, PLS-DA revealed distinct clusters of intestinal metabolites in Wuding chickens among different treatments (Fig. 5a). The number of up-regulated DAMs was 23, 44 and 80 in the HA, HB and HP individuals, respectively, compared to the HG samples; moreover, and 67, 104 and 108 metabolites were significantly reduced in the HA, HB and HP groups, respectively (Fig. 5b). Four metabolites were significantly more abundant in all three TCM-supplemented groups compared to the HG individuals, namely LPA 18:2, 1-oleoyl-LPA, allantoin, and an unknown metabolite (Fig. 5c). In contrast, a total of 44 metabolites were shown to be reduced in all three TCM-supplemented groups, including several dipeptides and tripeptides (Fig. 5c).

Gut microbiota of wuding chickens

Based on PacBio sequencing, a total of 5,067 bacterial OTUs were identified from the Wuding chicken gut microbiota, belonging to 15 phyla, 27 classes, 43 orders, 91 families, 244 genera, and 476 species. The rarefaction and species accumulation curves also showed a trend towards a plateau (Fig. S3), suggesting that the dataset presented here may reflect an intact Wuding chicken gut microbiota. Since the gut microbiota was obtained by PacBio sequencing, the annotation of OTUs at the species level was 87.86 % (Fig. S4a). Bacteroidaceae (15.8 %) was the most dominant bacterial family in the gut microbiota of the Wuding

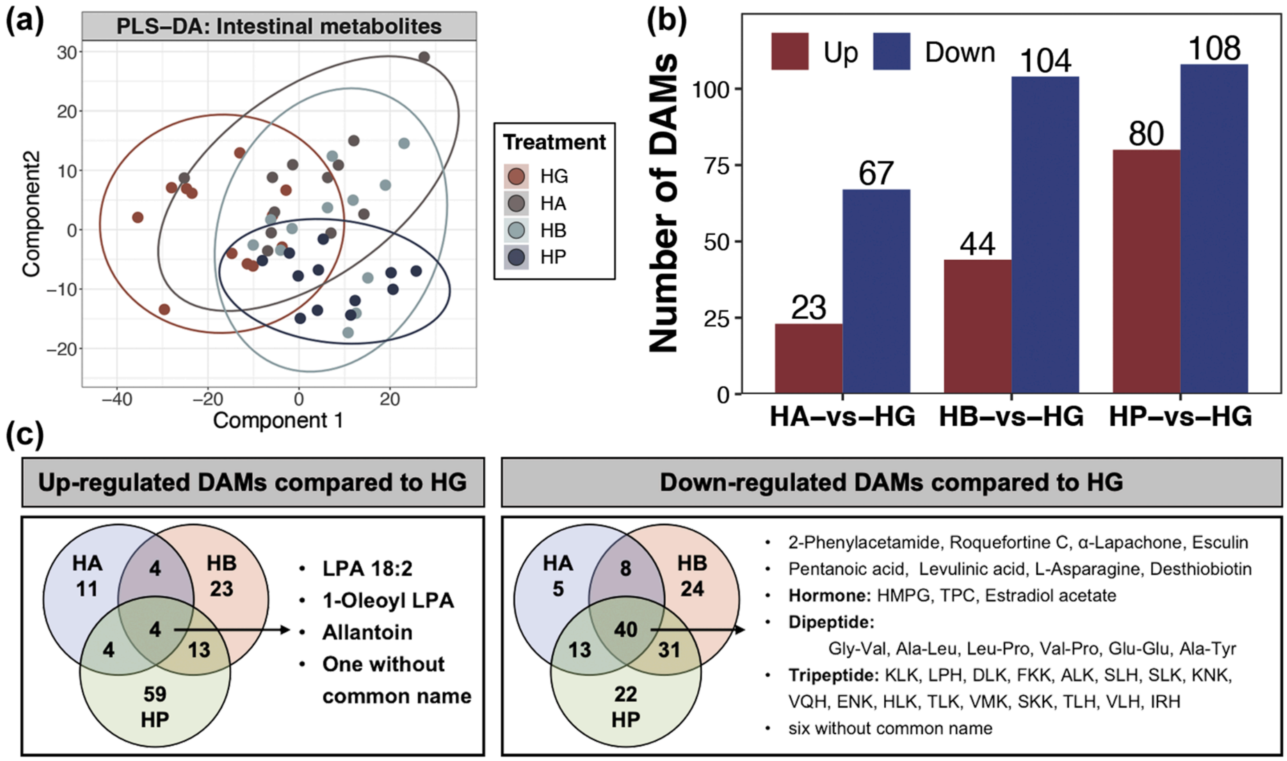

Fig. 5. (a) Partial least squares discriminant analysis (PLS-DA) showing the variations in intestinal metabolites of HDF Wuding chicken with and without dietary TCM. (b) Number of differentially abundant metabolites (DAMs) between the intestines of HDF Wuding chicken with and without dietary TCM. (c) Venn diagram showing the common up- and down-regulated DAMs between different comparisons.

chickens, followed by Oscillospiraceae (9.8 %), Muribaculaceae (7.03 %) and Lachnospiraceae (6.6 %) (Fig. 6a). None of the calculated alpha diversity indices showed significant differences between the different treatments (Tukey's HSD test, $p > 0.05$, Fig. S4b). PCoA based on Bray-Curtis distance also showed limited variation in the gut microbiota composition between treatments (Fig. 6b). In all three TCM-added groups, the relative abundance of *Microbacter margulisiae* in the gut microbiota was significantly reduced compared to the HG individuals (Wilcox rank sum test, $p < 0.05$, Fig. 6c). In contrast, the relative abundance of *Duodenibacillus* sp. 003472385 was significantly increased in the HA and HB samples compared to the HG counterparts (Wilcox rank sum test, $p < 0.05$, Fig. 6c). In addition, *Phocaeicola salanitronis* and *Phascolarctobacterium faecium* were also more abundant in the gut microbiota of the HB samples compared to the HG individuals (Wilcox rank sum test, $p < 0.05$, Fig. 6c). Furthermore, the gut microbiota of the Wuding chickens exhibited a high abundance of genes associated with SCFAs production and amino acid metabolism, particularly acetate production (Fig. 6d) and amino acid biosynthesis (Fig. S5).

Associations of anti-oxidative indices with liver and intestinal variables in wuding chickens

First, the abundance of genes related to SCFAs production in the gut microbiota showed significant negative correlations with the expression levels of genes related to cholesterol synthesis in the liver of the Wuding chickens (Spearman correlation, $p < 0.05$, Fig. 7). These correlations were more obvious for the genes of *acyP*, and *ackA*, all of which respond to acetate and propionate production and were significantly enriched in the gut microbiota of high HDF Wuding chickens with dietary TCMs (Fig. 6d). The contents of SCFAs in the intestine of the Wuding chickens were further measured, and the results showed higher acetate and propionate concentrations in the high HDF Wuding chickens with dietary TCMs compard to the HG individuals, especially for the HB group (Fig. 7). In addition, cholesterol levels in both the serum and liver of the Wuding chickens were significantly lower in the individuals fed TCMs compared to the HG counterparts, which was increased by HDF (Fig. 7). Furthermore, all the down-regulated cholesterol synthesis genes were positively correlated with MDA in the serum and liver of Wuding chickens, and some of them were negatively correlated with other antioxidant indices (Spearman correlation, $p < 0.05$, Fig. 7). These results indicated that dietary TCMs for HDF Wuding chickens could improve their reduced antioxidant capacity via the inhibition of cholesterol synthesis in the liver mediated by the improvement of acetate production in the intestine.

On the other hand, the expression levels of cholesterol synthesis genes in the liver of the Wuding chickens did not show obvious correlations with the potential key liver microorganisms (Fig. S6) or intestinal DAMs (Fig. S7). In contrast, significant positive correlations were found between the key gut bacterial species and tripeptides (Spearman correlation, $p < 0.05$, Fig. 7), which were significantly reduced in the HDF Wuding chicken with dietary TCMs (Fig. 5c and 6c). Several significant correlations were then observed between these gut tripeptides and liver microorganisms, with significant differences in relative abundance between the HDF Wuding chickens with and without dietary TCM (Spearman correlation, $p < 0.05$, Fig. 7). Several correlations between these key liver microorganisms and antioxidant indices were also identified (Spearman correlation, $p < 0.05$, Fig. 7). These correlations suggested that the dietary TCMs for HDF Wuding chickens could improve their reduced antioxidant capacity by another mechanism. The dietary TCMs altered the abundance of key species in the gut microbiota of the Wuding chickens, thereby regulating intestinal amino acid metabolism, which further affected key liver microorganisms and then altered the antioxidant capacity of the chickens.

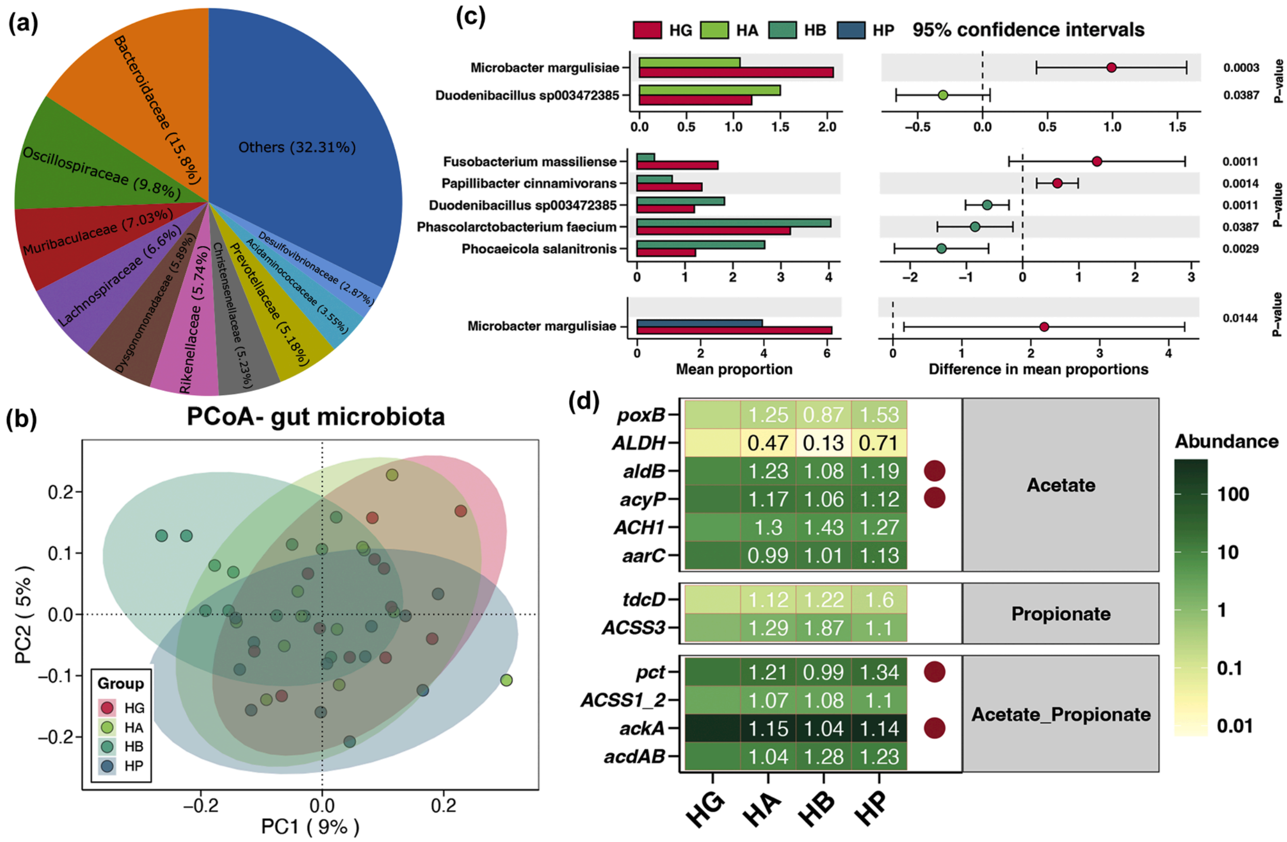

Fig. 6. (a) Pie chart showing the relative abundance of major bacterial families in the gut microbiota of Wuding chicken. (b) Principal coordinate analysis (PCoA) reveals the variations in the gut microbiota of HDF Wuding chicken with and without dietary TCMs. (c) Species with significant difference in relative abundance (WIlcox rank sum test, $p < 0.05$) in the liver of HDF Wuding chicken with and without dietary TCMs. (d) Heatmap showing the fold change of genes involved in the synthesis of short-chain fatty acids in the gut microbiota of HDF Wuding chickens with and without dietary TCMs. Values in blocks represent the fold change of each gene in the gut microbiota of HDF Wuding chickens with dietary TCMs compared to those of HG individuals. Red dots represent significant differences in the abundance of the corresponding gene between different groups (ANOVA, $p < 0.05$).

Discussion

HDF has been reported to induce oxidative stress in livestock farming, producing large amounts of reactive oxygen species, damaging the cell membrane of immune cells, and causing lipid and protein damage (Li et al., 2019). Previous studies have shown that chickens kept at high stocking densities have reduced activities of key antioxidant enzymes such as SOD and catalase in tissues such as the liver, heart and muscle (Magnuson et, al.,2020; Miao et al., 2021; Hafez et al., 2022). Similar results were found in the present study, decreased levels of SOD and GSH-Px and increased MDA concentrations (Fig. 1), suggesting that HDF may attenuate the antioxidant capacity of Wuding chickens.

Adding appropriate TCMs to the feed has been considered as an effective technique to alleviate oxidative stress damage in broilers (Tao et al., 2021; Shi et al., 2022). In this study, the effects of three TCMs (*Artemisia annua*, Huanglian and proanthocyanidin) on compensating for the impaired antioxidant system under HDF were investigated. Artemisinin, the bioactive compound of *Artemisia annua*, not only has potent antimalarial effects but also has strong antioxidant functions (Samal et al., 2021). Berberine, the bioactive compound of Huanglian, is rich in antioxidants that can neutralize free radicals in the body, reduce oxidative damage, protect cells from damage, and delay the aging process (Purwaningsih et al., 2023). Grape oligomeric proanthocyanidin, the bioactive compound of the three TCM used in the study, is an effective scavenger of reactive oxygen species and is currently recognized as one of the most effective natural antioxidants in scavenging free radicals in the body of animals (Nie et al., 2023). The results of this study indicate that all the tested compound can alleviate the antioxidant capacity in HDF Wuding chickens by increasing the levels of antioxidant indices in both the liver and serum (Fig. 2). Furthermore, proanthocyanidin showed the greatest efficacy among these three investigated CTMs in maintaining antioxidant defenses in the chickens at high stocking densities. Transcriptomics showed that the cholesterol biosynthesis pathway exhibited consistent downregulation across all three TCM-supplemented groups. Rauchbach et al. (2022) suggested that the hepatic cholesterol overload induces oxidative stress through redox imbalance and pro-inflammatory signaling cascades. In the present study, TCMs improve liver oxidative stress of HDF broilers may through inhiation of cholesterol biosynthesis.

The liver has traditionally been considered a sterile organ, but recent studies have challenged this view by demonstrating the presence of microorganisms in the liver of various animals (Zilber-Rosenberg and Rosenberg, 2008). A study in dogs found bacteria in the liver parenchyma of healthy animals, suggesting that the liver may harbor its own microbiome (Niza et al., 2004). In cattle, a bacterial community was observed in the liver parenchyma with Proteobacteria, Firmicutes and Actinobacteria being the dominant phyla (Asakura et al., 2022). Research in mice also revealed a microbial population in the liver dominated by Bacteroidetes, Firmicutes and Proteobacteria (Broderick and Nagy, 2022). Understanding the functions of the liver microbiome is an important area of research, as it may provide insights into the development and management of various liver-related diseases (Pirola et al., 2022). Some bacteria present in the liver may possess metabolic capabilities that could influence liver function and homeostasis, but the specific metabolic roles of the liver microbiome remain largely unknown (Jafarpour et al., 2020). In this study, a relationship between the liver

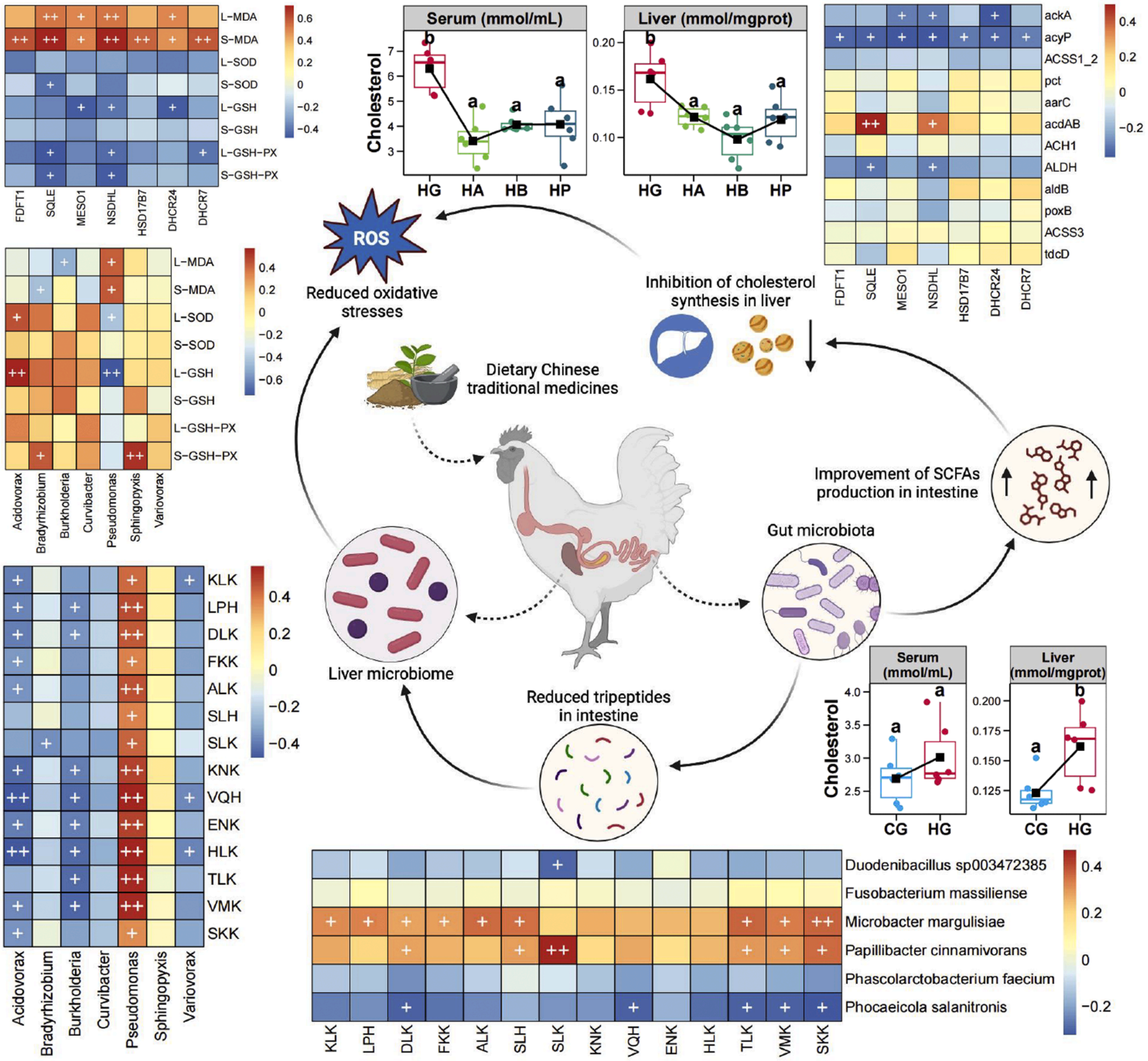

Fig. 7. Speculative mechanisms of the improvement of antioxidant capacity in HDF Wuding chicken with dietary TCMs. Box plots showing the variations of cholesterol levels in serum and liver as well as SCFAs contents in intestine of Wuding chicken among different groups. Different lowercase letters above the boxes of each sub-figure represent significant differences between different groups based on Tukey's HSD test ($p < 0.05$). Heatmap showing the correlations between different variables. The colour of the blocks represents the correlation coefficient, and "+" and "++" represent the p-value of the Spearman correlation less than 0.05 and 0.01, respectively.

microbiome and antioxidant defences was revealed, particularly for the increased *Pseudomonas* associated with weak liver antioxidant capacity (Fig. 7). *Pseudomonas* has been reported to use multiple virulence factors such as pyocyanin (Rada et al., 2011), pyocins (Chang et al., 2005), quinolone signalling molecules (Abdalla et al., 2017), and arachidonic acid (Saliba et al., 2006) to generate reactive oxygen species that induce oxidative stress in host cells. Interestingly, we found an enriched liver genus *Bradyrhizobium*, in the TCMs groups, which has beneficial effects on animals. The possible mechanism is that *Bradyrhizobium* can produce heme oxygenase and reduce oxygen free radicals (Mohammadi et al., 2001). Nevertheless, it is important to note that the existence and functional significance of the liver microbiome is still under investigation, and more research is needed to fully understand the complex interactions between microorganisms and the liver in different animal species.

While some research has focused on the effects of TCM supplementation on antioxidant function in high-density broiler management (Sugiharto, 2022), the specific mechanism underlying this effect is rarely reported. The gut is the direct site of action of CTM feed additives, and the liver is one of the major organs responsible for oxidative stress in poultry. The gut-liver axis in chickens is a complex and dynamic system that can affect the overall health and well-being of the bird (Hu et al., 2024b). In particular, the gut-liver axis is an integral part of the antioxidant system in animals, with significant implications for the management of oxidative stress (Xue et al., 2021). Commensal bacteria in the gut contribute to the maintenance of the muco-epithelial barrier by stimulating the production of mucus and antimicrobial peptides (Thomas et al., 2021). Disruption of this barrier can lead to increased intestinal permeability, allowing harmful substances to enter the liver and exacerbating oxidative stress (Rodrigues et al., 2024). In addition, changes in the composition of the gut microbiota can disrupt bile acid metabolism, leading to changes in bile acid profiles that can affect liver function and cholesterol homeostasis (Hu et al., 2022). The liver is also affected by compounds produced by the gut microbiota, such as SCFAs, which play a role in liver health and function (Aleksandra et al., 2023). These previous reports have highlighted the importance of a healthy gut microbiota for liver function and antioxidant capacity in chickens.

In this study, a pathway for the alleviation of antioxidant capacity in

HDF Wuding chickens by dietary CTMs was uncovered, where gut microbiota synthesized acetate and subsequently inhibited hepatic cholesterol synthesis (Fig. 7). Similar to the present study, a previous study found an inverse relationship between cholesterol levels and the activities of the antioxidant enzymes SOD and GSH-Px (Zamora-Ginez et al., 2019). In another study on aging laying hens, higher liver cholesterol was observed in older birds, which also had lower antioxidant enzyme activities (Gu et al., 2021). These findings suggest that higher hepatic cholesterol levels are associated with increased oxidative stress and reduced antioxidant enzyme activities. In addition, the regulation of cholesterol metabolism by SCFAs produced by the gut microbiota has previously been uncovered. SCFAs, particularly acetate, can activate enterocytes to secrete hormones into the bloodstream, which are then transported to the liver and regulate cholesterol metabolism (Jia et al., 2021). Meanwhile, SCFAs can also enter the liver via the portal vein and directly affect cholesterol metabolism by modulating the AMPK-PPARγ pathway (Sun et al., 2022). In conclusion, SCFAs produced by the gut microbiota play a critical role in regulating cholesterol metabolism in the liver via the gut-liver axis (Pant et al., 2023). The functional genes in the gut microbiota determine SCFA production and the addition of CTMs can modulate them in HDF Wuding chickens, indirectly affecting liver cholesterol levels and associated antioxidant defenses. To discuss this in further detail, CTMs enhanced the synthesis of SCFAs, especially acetate, in gut microbiota, which further transfer to the liver and lead to the reduction of cholesterol synthesis by downregulating the expression of related genes, and finally, the reduced cholesterol level alleviates the antioxidant system in Wuding chickens under HDF (Fig. 7).

The results of this study further linked the *Pseudomonas* in chicken liver to the tripeptides in the gut, which could be produced by specific gut bacteria such as M. *margulisiae* and *P. cinnamivorans* (Fig. 7). M. *margulisiae* is a propionigenic bacterium with propionic acid as a metabolic end product (Sanchez-Andrea et al., 2014), and *P. cinnamivorans* is an interesting anaerobic bacterium capable of degrading certain plant-derived aromatic compounds (Defnoun et al., 2000). While potential associations between these gut bacteria and tripeptides have been uncovered, there is no direct evidence that these bacteria are capable of producing tripeptides. Further research would be needed to determine whether tripeptide production is indeed one of the metabolic capabilities of these gut bacteria. Furthermore, the liver microbiome is closely linked to the gut microbiota, as the liver receives a significant proportion of metabolites from the gut via the blood supply (Victor and Quigley, 2016). The gut microbiota plays a crucial role in the production of various bioactive peptides, which can regulate gut hormones and modulate neurotransmitters in the host (Covasa et al., 2019). In addition, tripeptides produced by the gut microbiota can exhibit antioxidant and anti-inflammatory properties, which may contribute to the overall health of the host (Tsafack et al., 2022). Based on the above findings, a potential mechanism by which TCM supplementation alleviates antioxidant oxidative stress in chickens under HDF have been proposed. The supplementation of TCMs reduced in the tripeptides in the intestine by regulating the gut microbiota, which further transferred to the liver and inhibited the live microbiome, particular *Pseudomonas*, which can directly reduced the oxidative stress (Fig. 7). To the best of our knowledge, the present study is the first to investigate the potential association between gut microbiota-produced tripeptides and the liver microbiome. Furthermore, the direct production of tripeptides by the gut microbiota has only been demonstrated *in vitro*, and further *in vivo* studies are required to fully understand their physiological roles (Portune et al., 2016).

Conclusion

This study revealed reduced antioxidant capacity in HDF Wuding chickens and the beneficial effects of dietary TCMs in alleviating this inhibition. All three TCMs measured were shown to have the ability to alleviate antioxidant capacity in HDF Wuding chickens, with proanthocyanidin being the most effective. First, TCM supplementation significantly increased the production of SCFAs in the intestine of the HDF Wuding chickens, especially acetate, which inhibited cholesterol biosynthesis in the liver by down-regulating the expression of related genes. Second, the gut microbiota composition of the HDF Wuding chickens was significantly altered by dietary TCMs, with a reduction in the relative abundance of M. *margulisiae*. Changes in the gut microbiota can contribute to the reduced production of bioactive tripeptides in the gut, which regulated the composition of the liver microbiome, particularly reduced *Pseudomonas*, through the gut-liver axis response. Taken together, inhibition of cholesterol synthesis and reduced *Pseudomonas* in the liver ameliorated the reduced antioxidant defenses in HDF Wuding chickens. These findings provide a novel insight into the role of the gut-liver axis in the physiological response of broilers and highlight the effectiveness of the application of TCMs in broiler management. In addition, this study provides an explanation of the mode of action of TCMs from the perspective of modern science. Future research should explore the long-term effects of TCM supplementation in high-density chicken breeding and its benefits for poultry production to support potential commercial applications.

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Key R&D Program of China (No. 2022YFD1601905), Yunnan Agricultural Joint Special Project (No. 202401BD070001-005), China Agriculture Research System (CARS-40-S25), the Young Talent Project of the “Xing Dian Talent Support Program” of Yunnan Province in 2023 (XDYC-QNRC-2023-0403), the Industrial Innovation Talent Project of the “Xing Dian Talent Support Program” of Yunnan Province in 2022 (XDYC—CYCX-2022-0029), the Program of Anhui Provincial Key Laboratory of Livestock and Poultry Product Safety Engineering (XM2401), and Key research and development project of Anhui province (No. 2023n06020002) .

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105015.

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